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13. ABSTRACT (Maximum 200 Words)

We hypothesized that genes that are differentially expressed as a result of the decreased IGF-I receptor gene expression seen in metastatic prostate cancer contribute to prostate cancer progression, and include metastasis-regulating genes that could constitute valuable diagnostic markers or therapeutic targets. We initially proposed three specific aims: 1) Identification of differentially expressed genes in isogenic metastatic vs. non-metastatic prostate epithelial cells; 2) Identification of proteins that are differentially secreted in these cell lines, and 3) Assessment of the differential expression of these genes and proteins in laser-microdissected samples. We have used microarray gene profiling to characterize differentially expressed genes and have used SELDI-TOF mass spectrometry to identify proteins that are differentially secreted into conditioned media. In the last year, we obtained initial data suggesting that elevated IGF-I receptor expression controled survival in adult human male serum, which may explain the relationship between IGF action and metastasis. We proposed to take advantage of these new findings by investigating the molecular mechanisms underlying this effect and the cell-surface molecules expressed in metastatic and non-metastatic cells responsible for the differential sensitivity to serum, and requested a change in the statement of work. Unfortunately, these findings were not repeatable with subsequent batches of human serum, so that we have obtained a no-cost extension through 2/06 to readdress the original specific aims.

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Insulin-like growth factor; gene profiling, protein profiling

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Table of Contents

Introduction: The insulin-like growth factor (IGF) signaling system plays an important role in the initation and progression of prostate cancer. High circulating levels of IGF-I are associated with an increased risk of developing prostate cancer, and constitutive expression of IGF-I in the prostate epithelium of transgenic mice results in neoplasia. The actions of IGF-I are mediated through activation of the IGF-I receptor, a transmembrane tyrosine kinase that is highly expressed in normal prostate epithelium and immortalized prostate epithelial cell lines. A significant *decrease* in IGF-I receptor expression is seen in metastatic prostate cancer cell lines, human metastases, and metastatic lesions from transgenic mouse models of prostate cancer. Retroviral re-expression of the IGF-I receptor in metastatic prostate cancer cells reduces their tumorigenicity and metastatic potential. These data suggest that, while IGF-I action may contribute to the initiation of prostate cancer, a subsequent loss of IGF responsiveness secondary to reduced IGF-I receptor expression is necessary for progression to advanced disease. This requirement for decreased IGF-I responsiveness may reflect the ability of the activated IGF-I receptor to exert differentiative as well as proliferative effects. Our hypothesis is that genes that are differentially expressed as a result of the decreased IGF-I receptor gene expression seen in metastatic prostate cancer contribute to prostate cancer progression. Specifically, IGF-I receptor target genes may include metastasis-promoting or suppressing genes that could constitute valuable diagnostic markers or therapeutic targets. To evaluate this hypothesis, we proposed three specific aims: 1) Identification of a select subset of genes that are differentially regulated in otherwise isogenic metastatic vs. non-metastatic prostate epithelial cells that differ solely in the level of expression of the IGF-I receptor; 2) Identification of proteins that are differentially secreted in the cell lines used in aim 1, and 3) Assessment of the differential expression of these genes and gene products in laser-microdissected samples from normal prostate, adenocarcinoma, and metastatic lesions. The studies of specific aim 1 employ microarray gene profiling of metastatic prostate epithelial cells and their non-metastatic counterparts that are re-expressing the IGF-I receptor from a retroviral construct. The studies of aim 2 employ surface-enhanced laser desorption-ionization/time-of-flight (SELDI-TOF) mass spectroscopy to identify proteins that are differentially present in the conditioned media of the two cell types utilized in aim 1. Subsequent tandem mass spectroscopy analyses will be performed to generate proteolylic cleavage patterns or peptide sequences for gene identification. The studies of specific aim 3 will evaluate the expression of IGF-I receptor target genes in a series of human clinical samples using quantitative real-time RT-PCR analyses. The proposed studies address a critical aspect of prostate cancer, i.e., factors that contribute to the development of advanced disease. The studies underway employ an innovative, integrated, gene and protein profiling approach in a novel, carefully defined and highly controlled model system to identify genes and gene products that regulate metastasis. In the previous funding year, we obtained initial evidence that IGF-I receptor expression controled survival in human adult male serum, a novel finding that suggested that IGF action may regulate the initial step in the metastatic process, i.e., intravasation from the primary tumor. These data were extremely exciting in light of recent studies highlighting the critical role of intravasation in cancer progression (1-3), and we obtained permission to change the statement of work to pursue these findings. In subsequent studies, however, we were eventually unable to consistently replicate these findings with additional batches of male human serum or plasma. For these reasons, we have obtained a no-cost extension through 2/06 in order to readdress the original aims. Our progress with respect to the original aims is described below.

Body: The approved statement of work included three tasks that were to be initiated in the first 24 months of funding. These tasks were in support of aims 1 and 2 of the proposed project. These will be discussed in turn.

Task 1: Completion of microarray analysis of genes differentially expressed in LISN and LNL6 cells that express different levels of IGF-I receptor and which are, respectively, non-metastatic and metastatic in nude mouse xenografts. We have completed this analysis using three independent RNA preparations from each cell line grown in defined medium with 5% FBS and have analyzed each sample using triplicate arrays that each contain >12,000 sequence-verified, non-redundant human cDNA clones. Data have been analyzed by accepted means of normalization, statistical verification and false-discovery rate analyses. These data demonstrate that there are specific genes that are constantly differentially expressed in LISN and LNLG cells. We have recently acquired a rotary cell culture system for three-dimensional (3-D) cultures of cells in a microgravity

environment. This NASA-designed apparatus had been used to propagate cells under conditions that alter them to form 3-D structures that may be more similar to the in vivo situation (4, 5). Indeed, a recent report has shown that tumor cell lines grown under microgravity conditions display a phenotype that is much more similar to that of clinical samples in situ than cells grown in monolayer cultures (6). This system has been used in several previous studies to grow prostate cells in particular (7-10). We have now employed this novel culture system to repeat the array analysis that constituted task 1, since we feel that the data obtained will be significantly more relevant than that obtained with standard monolayer culture. These gene-profiling studies are currently being completed with latest-generation whole-genome CodeLink arrays and will be detailed in the final report.

Task 2: SELDI-TOF analysis of conditioned media from M12-LISN and M12-LNL6 cultures. Cells were grown in defined media supplemented with 5% FBS under either standard tissue culture conditions or in a 3-dimensional high-aspect rotating wall vessel system that more closely mimics in vivo conditions. Cell lines and media-LNL6 and LISN were grown in RPMI 1640 (Invitrogen) supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin and 250ug/ml fungizone (Invitrogen). For the 2D experiments, cells were seeded into T-75cm2 flasks and conditioned media collected at 72hrs. For the 3D experiments, cells were seeded at 1X107 cells with cytodex (Sigma) beads at 5mg/ml. Cells were allowed to attach to the beads and media was changed after 5 days. Conditioned media was collected 72 hrs later. SELDI sample preparation-Samples were prepared one of two ways; 1) straight conditioned media was used without any precipitation or fractionation or 2) conditioned media was precipitated in cold acetone at -20 degrees Celsius and washed in 95% ethanol. The pellet was dissolved in 6M urea and concentrated in a Centriplus YM-50 to remove serum albumin. The flow-through was precipitated again as above and resuspended in 500mM Tris-HCl pH 7.4. SELDI chips (Ciphergen)-NP20 (normal phase) are used for general binding of proteins for analysis. The active spots contain silicon oxide that allows proteins to bind via serine, threonine, and lysine residues. WCX-2 (weak cation-exchange) arrays can be used to analyze molecules with a postive charge on the surface. The active spots contain weak anionic carboxylate groups that interact with the positive charges on the surface of the analyte. SELDI chip sample application/analysis- For the NP20 chips-1ug and 5ug samples were applied to the spots and placed in a humidity chamber for 5 min. The excess sample was removed and the spots were washed 2X with water. Spots were allowed to air-dry and EAM was added and spot air-dryed. For the WCX-2 chips-samples were equilibrated in binding buffer (100mM ammonium acetate pH 6.0) 3X for 5min in a humidity chamber. 10ul of conditioned media was applied overnight at 4 degrees Celsius in a humidity chamber. Spots were washed 5X with binding buffer and 2X with water. EAM was applied while spots were still moist. Chips were analyzed at 220, 250 and 270 laser intensity on a Ciphergen PBS-II system.

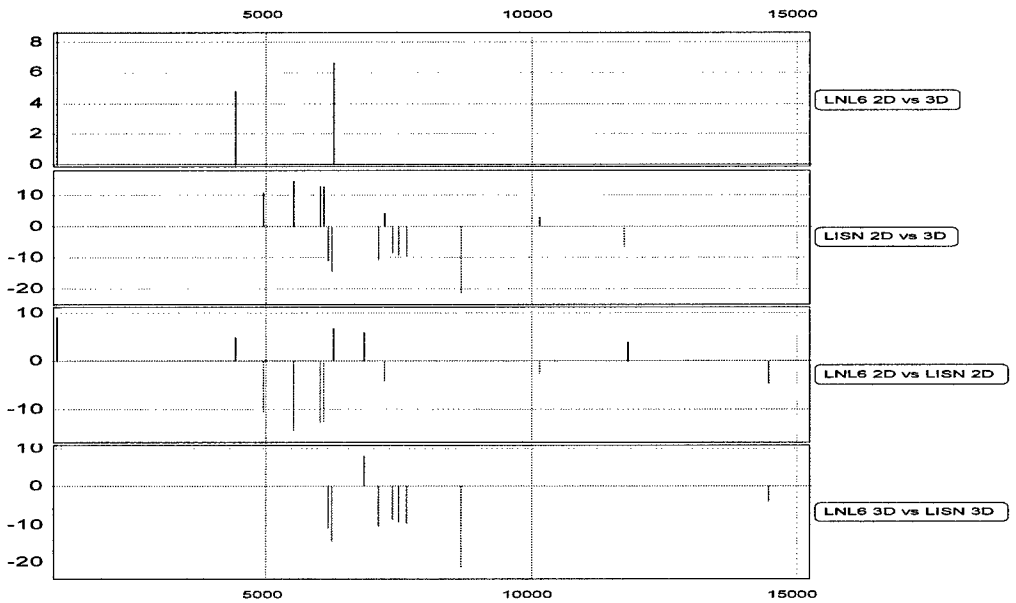

Figure 1. Difference spectra of LISN and LNL6 cells grown in 2-D and 3-D culture analyzed on NP-20 chips. 3-15 kDa range is shown.

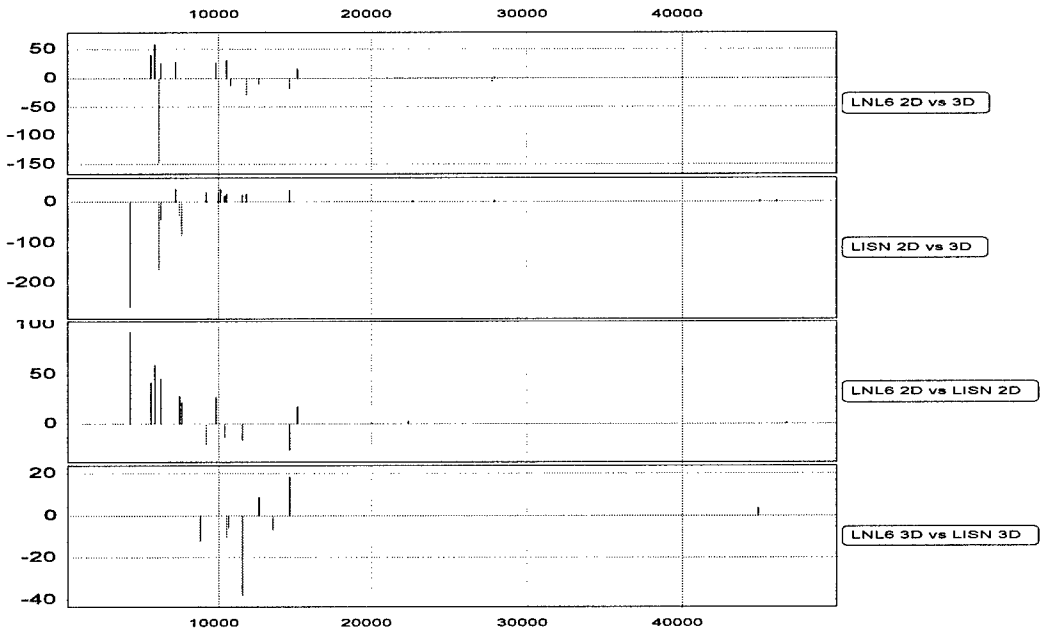

Figure 2. Difference spectra of LISN and LNL6 cells grown in 2-D and 3-D culture analyzed on NP-20 chips. 5-50 kDa range is shown.

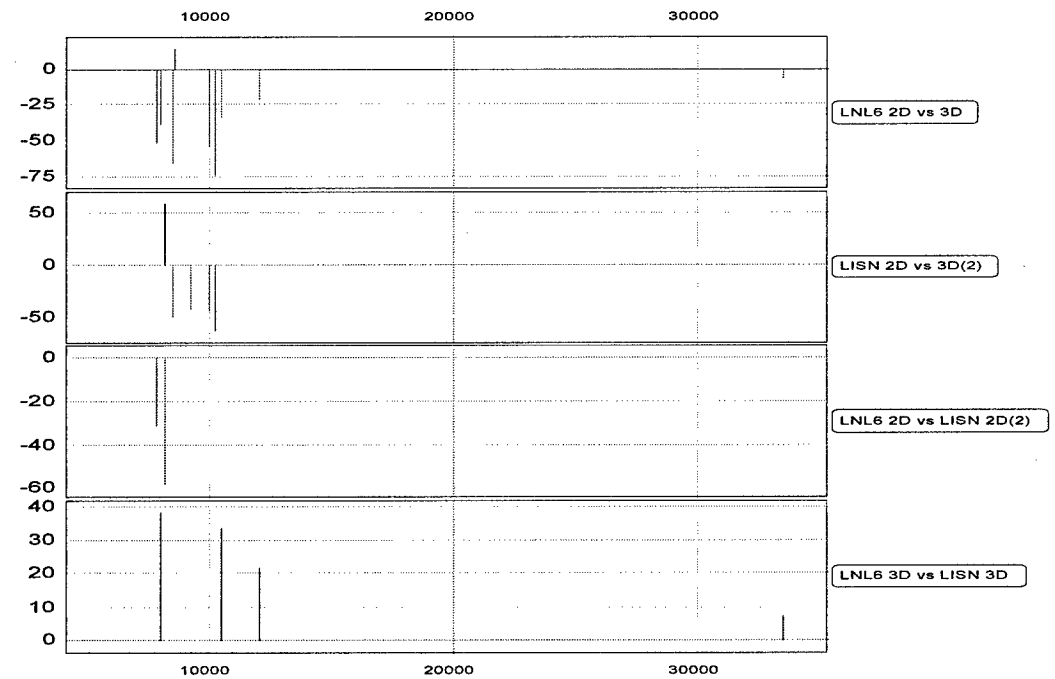

Figure 3. Difference spectra of LISN and LNL6 cells grown in 2-D and 3-D culture analyzed on WCX2 chips. 3-15 kDa range is shown.

SELDI-TOF profiling revealed a discrete set of proteins in the 4-25 kDa range that were differentially present in the CM of the LNL6 and LISN lines grown under 2-dimensional versus 3-dimensional conditions, as well as proteins that were differentially present in CM of LNL6 and LISN cells under either culture condition. Some of these proteins were differentially expressed between the different lines under both conditions. Thus, changes in IGF-I receptor expression of the degree characteristic of metastatic versus non-metastatic prostate cancer cells are associated with alterations in the secreted protein profile.

Task 3: Generation of probes for Northern and/or RPA analysis and verification of differential gene expression in M12-LISN and M12-LNL6 cells. We have begun to design primers of generation of cDNA probes for some of the robustly differentially expressed genes identified in the array studies of task 1, but will first determine which of these are differentially expressed in these cell lines grown in 3-D culture before proceeding further.

Tasks 4-8 concerned the evaluation of differentially expressed genes and proteins in clinical samples. We will initiate these studies following identification of fifferentially secreted proteins by tandem MS/MsS

Key research accomplishments:

- Demonstration of differential gene expression profiles in M12-LISN and M12-LNL6 cells expressing different levels of IGF-I receptor.

- Identification of specific molecular weight species that are differentially secreted by metastatic and non-metastatic prostate cancer cells under 2-D and 3-D culture conditions.

Reportable outcomes: (supported in part by this award)

- Abstract describing SELDI-TOF analyses of LISN and LNL6 cells in 2-D and 3-D culture presented at 86th Annual Meeting of the Endocrine Society in New Orleans, LA, June, 2004. Denley, A., Carroll, J.M., Nagalla, S.R., and Roberts, C.T., Jr., Proteomic analysis of IGF-regulated proteins in prostate cancer cells.

- Research article describing interactions between IGF and androgen receptor signaling in LISN and LNL6 cells and derivatives. Plymate, S.R., Tennant, M.K., Culp, S.H., Woodke, L., Marcelli, M., Colman, I., Nelson, P.S., Carroll, J.M., Roberts, C.T., Jr., and Ware, J.L. Androgen receptor (AR) expression in AR-negative prostate cancer cells results in differential effects of DHT and IGF on proliferation and AR activity between localized and metastatic tumors. Prostate 61: 276-290 (2004).

- Research article describing effect of saw palmetto extract on IGF signaling in P69 prostate cell line parent of LISN-LNL6 system accepted for publication. Wadsworth, T.L., Carroll, J.M., Roberts C.T., Jr., and Roselli, C.E. Saw palmetto extract suppresses IGF-I signaling and induces SAPK/JNK phosphorylation in human prostate epithelial cells. Endocrinology 145: 3205-3214 (2004).

- Idea Development Award application on androgen receptor regulation of IGF-I receptor expression funded by USAMRMC, CDRMP, PCRP, 12/04.

Conclusions: We have shown that differences in IGF-I receptor gene expression that are sufficient to alter metstatic capacity are sufficient to alter gene expression and secreted protein expression profiles in monolayer and in 3-D cell culture. There is an increasing appreciation that findings made in monolayer cell culture may not accurately reflect the molecular situation in vivo, as compared to what can be achieved with 3-D culture approaches (4, 11-13).

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Appendices: none